

## Interactions between Coronavirus Nucleocapsid Protein and Viral RNAs: Implications for Viral Transcription

RALPH S. BARIC,<sup>1</sup> GARY W. NELSON,<sup>2</sup> JOHN O. FLEMING,<sup>2</sup> ROBERT J. DEANS,<sup>2</sup> JAMES G. KECK,<sup>2</sup>  
NANCY CASTEEL,<sup>3†</sup> AND STEPHEN A. STOHLMAN<sup>2,3\*</sup>

*Department of Parasitology and Laboratory Practice, University of North Carolina School of Public Health, Chapel Hill, North Carolina 27514,<sup>1</sup> and the Departments of Microbiology<sup>2</sup> and Neurology,<sup>3</sup> University of Southern California School of Medicine, Los Angeles, California 90033*

Received 28 April 1988/Accepted 1 August 1988

**The interaction of the mouse hepatitis virus (MHV) nucleocapsid protein (N) and viral RNA was examined. Monoclonal antibody specific for N protein coimmunoprecipitated MHV genomic RNA as well as all six MHV subgenomic mRNAs found in MHV-infected cells. In contrast, monoclonal antibodies to the MHV E2 or E1 envelope glycoproteins, an anti-I-A monoclonal antibody, and serum samples from lupus patients did not immunoprecipitate the MHV mRNAs. Moreover, the anti-N monoclonal antibody did not coimmunoprecipitate vesicular stomatitis virus RNA or host cell RNA under conditions which immunoprecipitated all MHV RNAs. These data suggest a specific interaction between the N protein and the virus-specific mRNAs. Both the membrane-bound and cytosolic small MHV leader-specific RNAs of greater than 65 nucleotides long were immunoprecipitated only by anti-N monoclonal antibody. These data suggest that an N binding site is present within the leader RNA sequences at a site at least 65 nucleotides from the 5' end of genomic RNA and all six subgenomic mRNAs. The larger leader-containing RNAs originating from mRNA 1 and mRNA 6, as well as the MHV negative-stranded RNA, were also immunoprecipitated by the anti-N monoclonal antibody. These data indicate that the MHV N protein is associated with MHV-specific RNAs and RNA intermediates and may play an important functional role during MHV transcription and replication.**

Mouse hepatitis virus (MHV) is a member of the coronavirus group of animal viruses. MHV is an enveloped virus containing a helical nucleocapsid structure composed of a plus-sensed RNA molecule ( $5.5 \times 10^6$  daltons) and a nucleocapsid protein (N) of 60,000 daltons (37). The envelope contains a 180,000-dalton N-linked glycoprotein (designated E2) which functions in cell attachment and fusion and an O-linked matrix glycoprotein of 23,000 daltons (designated E1) (38).

During infection, virion RNA is initially transcribed by an early polymerase activity into a genome-sized negative-stranded RNA (9). In turn, the negative-stranded RNA is transcribed by a late polymerase activity into a full-length genomic RNA that is both bound to polysomes (9) and detected in EDTA-resistant nucleocapsid structures (34). Viral transcription is associated mainly with membrane fractions (9, 10). Six species of subgenomic mRNAs are synthesized during infection. They are arranged in the form of a nested set with common sequences derived from the 3' end of the genomic RNA that extend in the 5' direction for various distances (21, 34, 38). Therefore, each mRNA contains its own unique sequences plus all the sequences contained within the next smaller mRNA species. In addition, all viral mRNAs contain an identical leader sequence of approximately 72 nucleotides at their 5' end (21, 22). Analysis of MHV-infected cells has revealed the presence of small leader RNAs 65 to 84 nucleotides long which could serve a primer function by binding to specific sequences conserved at the intergenic regions in the negative-strand template. These leader sequences are derived from the 5' end of the genomic RNA and are probably joined to the

individual mRNAs by a unique leader-primed transcription mechanism (4-6, 21, 25, 32).

The amino acid sequence of N, deduced from the nucleic acid sequence, reveals that this protein is 455 amino acids long and highly basic (1, 33). Immediately after synthesis, the N protein is phosphorylated exclusively on serine residues. Phosphorylated N is associated with cellular membranes via a strong noncovalent interaction, presumably through association with the E1 protein (36, 39). A 140,000-dalton trimer of N has been described in nonreduced preparations of purified virions by using an RNA protein-overlay blot assay, suggesting that N protein may exist as a disulfide-linked multimer in the mature virion (29). The N protein is also the only RNA-binding protein in virions and is a major RNA-binding protein found in infected cells (29).

The N protein has a variety of functions in addition to serving as a structural component of the helical nucleocapsid (38). It also plays important roles in viral pathogenesis and replication, since anti-N monoclonal antibodies protect mice from lethal infection (27) and inhibit viral transcription *in vitro* (12); however, the function of N in viral transcription is unclear. In this report, we have examined the association of the N protein with MHV RNA by using an anti-N monoclonal antibody to immunoprecipitate the N-RNA complexes. In the accompanying article, we have examined the specificity of N-RNA interactions by a second technique using a direct RNA-protein binding assay (8, 15, 29, 35). Together, these data suggest that N protein-MHV RNA interaction is mediated by binding signals contained within MHV leader RNA sequences and suggest that this interaction is important in viral transcription.

### MATERIALS AND METHODS

**Virus and cells.** The A59 strain of MHV was used throughout the course of this study. Virus was propagated in DBT

\* Corresponding author.

† Present address: Department of Microbiology, Chicago College of Osteopathic Medicine, 1122 E. 53rd St., Chicago, IL 60615.

cells, a murine astrocytoma cell line, or in L2 cells as previously described (4, 22, 23). Cells were grown in 150- or 100-mm-diameter petri plates and infected at a multiplicity of infection of 1 to 5. For experiments using  $^{32}\text{P}_i$  to radiolabel MHV-specific mRNAs, infected cells were treated with 2  $\mu\text{g}$  of actinomycin D per ml at 2.5 h post-infection (p.i.) and labeled with 500  $\mu\text{Ci}$  of  $^{32}\text{P}_i$  (ICN Pharmaceuticals Inc., Irvine, Calif.) at 4.5 h p.i. Samples were harvested as described below at 7.5 to 8.5 h p.i. [ $^3\text{H}$ ]uridine (ICN) was added to actinomycin D-treated cells at 15  $\mu\text{Ci}/\text{ml}$  at 4.5 h p.i. For coinfection studies, the Indiana strain of vesicular stomatitis virus (VSV) was used at a multiplicity of infection of 5. Plates were infected with A59 as described above, incubated for 30 min at 37°C, and subsequently coinfecting with VSV for 1 h at 37°C. Radiolabeled purified virions were prepared as previously described (23).

**Cell lysates.** At 7.5 to 8.5 h p.i. the plates were chilled in an ice-water bath, the medium was removed, and the plates were washed twice with ice-cold phosphate-buffered saline. Cells were lysed by the addition of 3 ml of 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl per plate, 0.5% Nonidet P-40, and 200  $\mu\text{g}$  of phenylmethylsulfonyl fluoride per ml. After 10 to 15 min the cells were removed with a rubber policeman and the nuclei and large cell debris were pelleted by centrifugation at  $1,500 \times g$  for 5 min at 4°C. The supernatant was then adjusted to 10 mM EDTA and 200 mM ammonium acetate. After an additional centrifugation at  $15,000 \times g$  for 10 min, the supernatant was adjusted to 0.2% sodium dodecyl sulfate (SDS) before immunoprecipitation.

**Preparation of P100 or S100 subcellular extracts.** Preparations of subcellular fractions were as previously described (6). Briefly, cells were swollen in 0.5 $\times$  reticulocyte standard buffer (1 $\times$  reticulocyte standard buffer is 10 mM Tris [pH 7.4], 10 mM NaCl, 1.5 mM  $\text{MgCl}_2$ ) for 30 min at 4°C. The cells were disrupted by Dounce homogenization and centrifuged at  $1,000 \times g$  for 5 min. The nuclear pellet was discarded, and the membrane fractions (P100) were separated from the cytosol fraction (S100) by centrifugation at  $100,000 \times g$  for 90 min. Under these conditions over 90% of the ( $\text{Na}^+\text{K}^+$ )-ATPase and 5'-nucleotidase were present in the P100 fraction (6). Membrane fractions were suspended in 0.5 $\times$  reticulocyte standard buffer before immunoprecipitation. RNA in the immunoprecipitates was extracted and separated on 8 to 12% polyacrylamide gels before electrotransfer to Zeta-Probe paper (5).

**Immunoprecipitation.** Immunoprecipitations were carried out as previously described (14, 16, 36). In these experiments, anti-A59 monoclonal antibodies A.1.10, specific for N; A.3.10, specific for the E2 envelope glycoprotein; A.1.8, specific for the E1 glycoprotein; and 7.16.17, specific for I-A<sup>p</sup>, were used (14, 16, 18). Briefly, antibodies were incubated with the cell lysates for 30 min on ice, and then Formalin-fixed *Staphylococcus aureus* Cowan 1 was added. This mixture was incubated 45 min on ice and then washed twice in 10 mM Tris (pH 7.4) containing 150 mM NaCl, 0.5% Nonidet P-40, 0.2% SDS, and 200  $\mu\text{g}$  of phenylmethylsulfonyl fluoride per ml. The final pellets were suspended in 10 mM Tris (pH 7.4) containing 60 mM NaCl, 1 mM EDTA, and 1.0% SDS. The RNA was extracted with phenol and chloroform as previously described (4, 22) and precipitated overnight with ethanol at -20°C. RNA was treated with glyoxal and analyzed by electrophoresis on 1% agarose gels as previously described (4, 22). Radiolabeled nucleic acids were visualized by either fluorography or autoradiography (4, 22).

**Immunoprecipitation of the MHV leader RNAs.** Cultures of

infected and uninfected cells were lysed and incubated with a variety of antisera as described above. In addition, antiserum samples from systemic lupus erythematosus patients specific for the La, Rho, Sm, and ribonucleoprotein (RNP) complexes (kindly provided by Jack Keene, Duke University, Durham, N.C.) were used. After immunoprecipitation as described by Kurilla and Keene (20), the RNA was extracted, precipitated, and separated on 8 or 12% polyacrylamide gels containing urea (4). The RNA was electrotransferred to Zeta-Probe paper, prehybridized, and probed with a nick-translated leader cDNA probe (E1-L) or internal E1 sequences (E1-500). The E1-L cDNA probe encodes nucleotides 1 to 84 of mRNA 6, including the 72-nucleotide leader sequence, while E1-500 encodes the internal nucleotides 90 to 609 of mRNA 6 (4). The blots were washed and exposed to XAR-5 film in the presence of an intensifying screen at -70°C (4).

**Northern (RNA) blots.** For Northern analysis, RNA samples were electrophoresed on 0.8% formaldehyde-agarose gels and blotted by capillary diffusion to nitrocellulose (40). Bound RNA was hybridized to a  $^{32}\text{P}$ -labeled 1,360-base-pair fragment of a human gamma actin gene probe, which cross-hybridizes to mouse actin sequences (11). The hybridization probe was prepared to a specific activity of  $10^9$  cpm/ $\mu\text{g}$  by the method of Feinberg and Vogelstein (13).

**In vitro transcription of MHV strand-specific probes.** Recombinant plasmid pT7-2 11, containing sequences derived from MHV gene F (nucleotides 1 to 760), and plasmid pT7F82P (nucleotides 5 to 1149) (4, 32), containing sequences derived from MHV gene A, are oriented in the plus-sense configuration so that T7 polymerase-derived transcripts are identical to mRNA and capable of annealing with MHV negative-stranded but not plus-stranded RNA. Transcription was carried out in a 100- $\mu\text{l}$  reaction containing 2  $\mu\text{g}$  of linearized plasmid DNA; 40 mM Tris hydrochloride (pH 7.5); 6 mM  $\text{MgCl}_2$ ; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 1 mM each ATP, CTP, and GTP; 0.01 mM UTP; 100  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]UTP; 10 U of T7 RNA polymerase; and 40 U of RNasin. Reactions were incubated at 37°C for 1 h, and the radiolabeled products were separated from free nucleotides by chromatography through P-60 columns. Radiolabeled RNAs were mixed with hybridization buffer and incubated with the filter at 65°C for 24 to 36 h (5, 6).

**Detection of MHV negative-stranded RNA.** Intracellular RNA from MHV-infected cells was immunoprecipitated as described above, denatured by dimethyl sulfoxide-glyoxal or heat, and applied to nitrocellulose paper by using a dot blot unit (Bio-Rad Laboratories, Richmond, Calif.). The paper was baked at 80°C for 2 h, prewashed and prehybridized as previously described (4, 6), and then probed with plus-stranded RNA probes ( $2 \times 10^7$  cpm/ml) capable of annealing with MHV negative-stranded but not plus-stranded RNA or purified genomic RNA. The filters were hybridized at 65°C for 24 to 36 h in a solution containing 50% formamide, 10 mM sodium phosphate (pH 7.0),  $10 \times$  Denhardt solution, 1.83 mM EDTA,  $6 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 83  $\mu\text{g}$  of tRNA per ml, and 125  $\mu\text{g}$  of salmon sperm DNA per ml. After hybridization, the filters were washed with several changes of  $2 \times$  SSC containing 0.01% SDS and with  $0.2 \times$  SSC containing 0.01% SDS at 65 to 70°C. Finally, blots were treated with 0.5  $\mu\text{g}$  of RNase A per ml at room temperature in  $2 \times$  SSC for 5 to 15 min. Filters were exposed to XAR-5 film in the presence of an intensifying screen at -70°C.

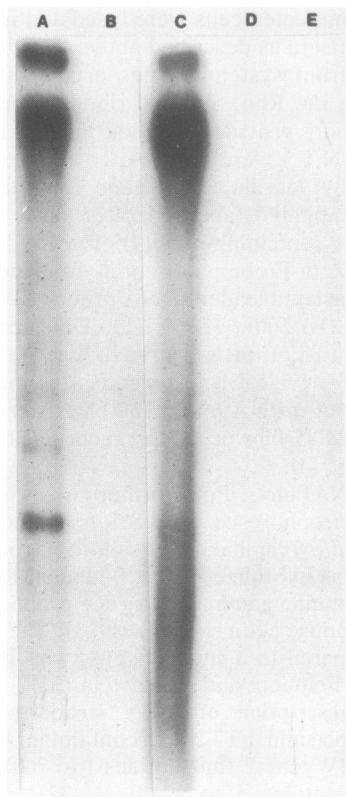


FIG. 1. Immunoprecipitation of RNA from pelleted MHV virions. Lanes: A, genomic-length RNA extracted from the pelleted virions with some contamination from mRNAs 5, 6, and 7; B, no RNA recovered in the absence of antibody; C, RNA of genomic length immunoprecipitated by using anti-N monoclonal antibody; D and E, no RNA recovered with anti-E1 or anti-E2 monoclonal antibodies.

## RESULTS

**Association of N protein with viral RNA.** Monoclonal antibodies specific for the N protein, the E1 envelope glycoprotein, and the E2 envelope glycoproteins of the A59 strain of MHV (16) were tested to determine whether a specific interaction between a particular viral structural protein and the MHV genomic RNA could be demonstrated by coimmunoprecipitation. Antibodies were incubated with concentrated  $^{32}\text{P}$ -labeled detergent-disrupted A59 virions. The RNA was extracted from the immunoprecipitated complexes and analyzed by electrophoresis on denaturing agarose gels. Anti-N monoclonal antibody immunoprecipitated MHV genomic RNA as part of an N protein-RNP complex (Fig. 1). No RNA was detected in immunoprecipitates formed by using the anti-E1, anti-E2, or irrelevant anti-major histocompatibility complex class II I-A<sup>p</sup> monoclonal antibodies. These data support previous studies indicating that N protein interacts with the genomic RNA to form a helical nucleocapsid structure (38) and demonstrate the feasibility of examining RNA-protein interactions by immunoprecipitation.

We next analyzed the RNA species immunoprecipitated with anti-N monoclonal antibody from lysates of  $^{32}\text{P}$ -labeled actinomycin D-treated MHV-infected cells. Anti-N monoclonal antibody immunoprecipitated not only genomic RNA (RNA 1) but, surprisingly, the other six virus-specific intra-

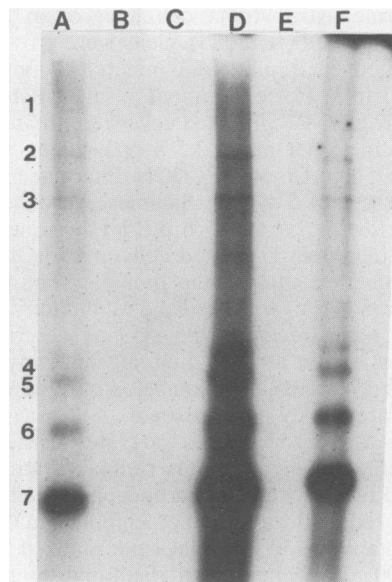


FIG. 2. Immunoprecipitation of RNA from MHV-infected cells. Lanes: A and F, controls showing the virus-specific RNA extracted from infected cells; D, all seven virus-specific RNAs immunoprecipitated by the anti-N monoclonal antibody; B, C, and E, immunoprecipitates obtained by using anti-E1, anti-E2, and anti-I-A<sup>p</sup> monoclonal antibodies, respectively. MHV mRNAs 1 through 7 are indicated on the left.

cellular RNAs as well (Fig. 2). Also, no virus-specific mRNAs were immunoprecipitated with monoclonal antibodies specific for either the E1 or E2 protein (Fig. 2). Immunoprecipitation of individual E1 or E2 mRNA from polyosomes was not detected because of the dissociation of the complexes with EDTA before immunoprecipitation (see Materials and Methods). In addition, no virus-specific RNA was immunoprecipitated with the antibody specific for the I-A<sup>p</sup> protein. These data suggest that all seven viral intracellular mRNAs exist as RNP complexes during infection.

The specificity of the immunoprecipitation of the intracellular virus-specific mRNAs by anti-N monoclonal antibody was further examined in experiments designed to clearly demonstrate specificity for MHV RNAs. First, cells were coinfectd with VSV and MHV in the presence of actinomycin D to determine whether the MHV N protein was associated with VSV RNA during coinfection as previously suggested (29). These experiments are critical, since VSV RNAs probably would be immunoprecipitated if nonspecific interactions occur during sample preparation. Coinfectd cells contained both MHV and VSV RNA (Fig. 3). The relative amounts of VSV and MHV RNA in the coinfectd cells varied slightly from experiment to experiment. Figure 3 shows the results of an experiment in which relatively equal amounts were present. More importantly, the MHV-specific anti-N monoclonal antibodies immunoprecipitated only MHV-specific mRNAs but not VSV RNA from the coinfectd cells.

Cells were also prelabeled overnight with [ $^3\text{H}$ ]uridine to label host cell RNAs. Label was removed, the cells were infected and treated with actinomycin D, and the intracellular RNAs were immunoprecipitated as described above. Control cells were infected, treated with actinomycin D, and radiolabeled with [ $^3\text{H}$ ]uridine during infection. Cells labeled during infection yielded only virus-specific mRNAs after

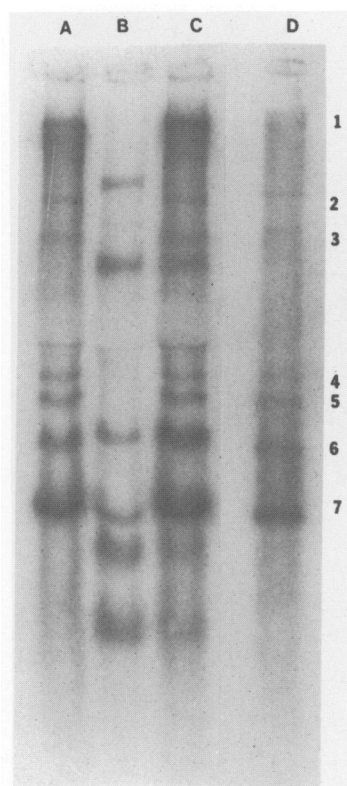


FIG. 3. Immunoprecipitation of MHV-specific RNA from cells coinfecting with VSV. Lanes: A, pattern of RNA extracted from MHV-infected cells; B, RNA extracted from VSV-infected cells; C, RNA extracted from cells coinfecting with both VSV and MHV. Note that most MHV mRNAs (numbered 1 through 7 on the left) can be detected. Lane D, RNA immunoprecipitated from cells coinfecting with VSV and MHV, demonstrating that only MHV mRNA is recovered by using the anti-N monoclonal antibody.

immunoprecipitation with anti-N monoclonal antibody. RNA immunoprecipitated from cells labeled before infection contained no host cell RNA species; however, a very small quantity of virus-specific mRNA, presumably derived from the turnover of [ $^3$ H]uridine during the infection, was detected (data not shown). Finally, RNA from infected and uninfected cells was immunoprecipitated with anti-N monoclonal antibody and examined for actin mRNA by Northern blot analysis. Similar amounts of actin mRNA were detected in infected and uninfected control cells; however, no actin RNA was detected in the immunoprecipitates from either infected or uninfected cells (data not shown). These data indicate that the MHV N protein is not associated with either host cell or VSV mRNA molecules and suggest that a specific interaction between the MHV N protein and viral RNA occurs during infection.

**Mapping the N binding site to a location within the leader RNA.** Immunoprecipitation of all the MHV genomic and subgenomic mRNAs suggested that the N binding site must reside within sequences common to each RNA. The 5' and 3' ends of all seven MHV RNAs are conserved because of the presence of a common leader RNA at the 5' end and the nested-set structure at the 3' end (21, 22). Previous results from our laboratories have also demonstrated that several small leader-containing RNAs of 65 to 84 nucleotides in length are derived from the 5' end of the viral genome and may function as primers in mRNA synthesis (4, 6). To

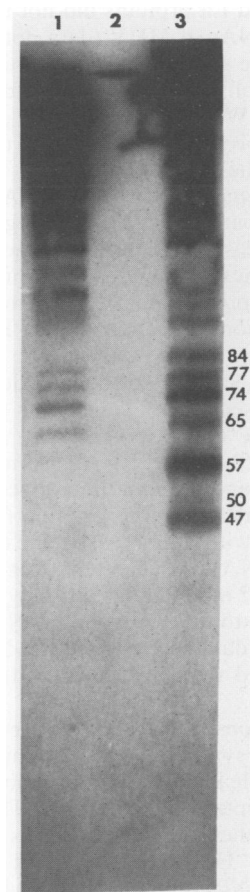


FIG. 4. Immunoprecipitation of MHV small leader-containing RNAs. Cultures of infected cells were lysed and immunoprecipitated with a variety of antisera directed against MHV structural proteins or antiserum obtained from lupus patients. The RNA protein complexes were extracted, and the nucleic acid was separated on 8% polyacrylamide gels. After electrotransfer to Zeta-Probe paper, the blots were hybridized with a leader-specific cDNA. Lanes: 1, RNA immunoprecipitated with anti-N; 2, immunoprecipitation with anti-E2; 3, total RNA extracted from MHV-infected cells. Nucleotide numbers are shown on the right.

determine whether the small leader RNAs of MHV bind N, we performed immunoprecipitations using monoclonal antibodies specific for the MHV N and E2 proteins and a number of serum samples from systemic lupus erythematosus patients containing antibodies specific for the La, Sm, RNP, and Rho proteins. These serum samples were tested because previous data have demonstrated that the VSV leader RNA is bound to the host La protein (20). Immunoprecipitated RNA from infected cells was separated on 8% polyacrylamide gels, transferred to Zeta-Probe paper and hybridized with a nick-translated leader-specific cDNA probe (E1-L). Anti-N monoclonal antibody immunoprecipitated the small MHV leader RNAs of 65 nucleotides or longer, but not the 47- or 50-nucleotide RNAs. It is unclear whether the 57-nucleotide leader RNA is precipitated by N (Fig. 4). A slight size difference (2 to 5 nucleotides) was detected in the 74-, 77-, and 84-nucleotide RNAs between the immunoprecipitated and nonprecipitated RNA. Although the exact reason for this is unclear, it was probably caused by endogenous or exonuclease activities, or both, present during immunoprecipitation. Antibodies with specificities for the MHV E1,

Rho, Sm, RNP, and La proteins did not immunoprecipitate the small leader RNAs of MHV (data not shown).

In addition to the small leader-containing RNAs, leader-containing RNAs of 100 nucleotides and larger were also detected (Fig. 4). We have previously demonstrated that specific, highly reproducible subsets of these small RNAs originate during the transcription (4). Furthermore, sequence analysis suggests that these RNAs are synthesized by transcriptional pausing in AU-rich regions or regions of secondary structure (4). To determine whether the N protein is also bound to these larger leader-containing RNAs, we separated immunoprecipitated RNA from MHV-infected cells under conditions which resolved RNAs 100 to 300 nucleotides long (4). These RNAs were hybridized with a radiolabeled cDNA specific for nucleotides 90 to 609 in the mRNA 6 (gene F) sequence. Transcripts of identical length were present in the anti-N precipitated and control RNA preparations (Fig. 5). These RNAs are identical in size to those previously reported on the replicative-intermediate (RI) RNA during mRNA 6 transcription (4). Similar results were also obtained with a gene A-specific cDNA probe (data not shown). No RNA was detected after immunoprecipitation with the anti-E2 monoclonal antibody. Since the only 5' sequences common to mRNA 6 and mRNA 1 are within the leader RNA, these data suggest that the N protein binds to sequences contained within or adjacent to the leader RNA sequences.

**Subcellular locations of N-bound leader-containing RNAs.** MHV leader RNA synthesis is discontinuous from mRNA synthesis, and the leader RNA(s) probably act in *trans* to prime viral transcription (4–6, 25, 32). The data described above suggest that the small leader RNAs are bound to N in an RNP complex. However, it is not clear whether a leader-containing RNP complex or a naked RNA molecule is released from the template to act in *trans* as a primer for subgenomic mRNA synthesis. Previous data have demonstrated that MHV leader RNAs are associated with the cellular membranes and cytoplasmic fractions in roughly equal molar ratios (6). To determine whether N is associated with the free leader RNAs, the subcellular location of the leader-RNP complexes was determined by separating the membrane (P100) and cytosolic (S100) fractions by ultracentrifugation as previously described (6). Subcellular fractions were immunoprecipitated with anti-N or anti-I-A monoclonal antibodies, and the small RNAs were separated by polyacrylamide gel electrophoresis. After transfer to Zeta-Probe paper, the blots were hybridized with a nick-translated leader-specific cDNA (E1-L) probe. Identically sized leader RNAs were present in immunoprecipitates and control preparations (Fig. 6), indicating that the small leader RNAs of MHV are associated with the N protein in both the membrane and cytosolic fractions. These data are consistent with previous data indicating that MHV leader-containing RNAs and the N protein are associated with the membranes of infected cells (6, 36).

**Analysis of N-function in viral transcription.** Anti-N monoclonal antibody inhibits the *in vitro* transcription of viral RNA, suggesting that the N protein is tightly associated with the transcription complex (12). To further clarify the role of N in viral transcription, we examined the interaction of N with the RI RNA. RI RNA consists of a genome-length negative-stranded RNA hydrogen bonded to nascent plus-strands representing newly transcribed RNA (5). Since little, if any, free negative-stranded RNA is present in infected cells (31), the presence of negative-stranded RNA in the immunoprecipitates would suggest that N was bound to the

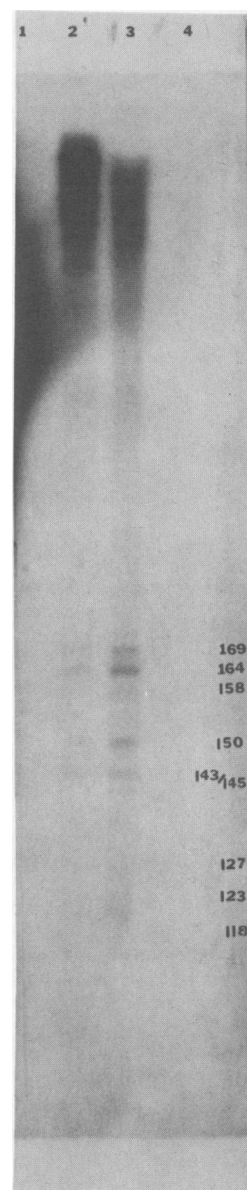


FIG. 5. N binding by leader RNAs originating during mRNA 6 transcription. Cultures of cells were lysed and immunoprecipitated with anti-N or anti-E2 monoclonal antibodies as described in Materials and Methods. The complexes were extracted and separated on 8% polyacrylamide gels under conditions which resolve small RNAs 100 to 300 nucleotides long. The RNA was electroblotted to Zeta-Probe paper and hybridized with a nick-translated cDNA clone spanning nucleotides 90 to 609 in the gene F sequence (6). Lanes: 1, anti-E2-immunoprecipitated RNAs from infected cells; 2, anti-N-immunoprecipitated RNAs from infected cells; 3, total infected-cell intracellular RNA; 4, total RNA from uninfected cells. Nucleotide numbers are shown on the right.

RNA of the RI complex. Therefore, lysates of infected and uninfected cells were incubated with either anti-N or anti-E2 monoclonal antibodies, immunoprecipitated, and probed for the presence of negative-stranded RNA. Studies in our laboratory and others indicate that strand-specific RNA probes are capable of detecting as little as  $10^3$  to  $10^4$  infectious units of virus and that *in vitro*-transcribed plus-sense RNAs do not bind to purified genomic RNA (41). The

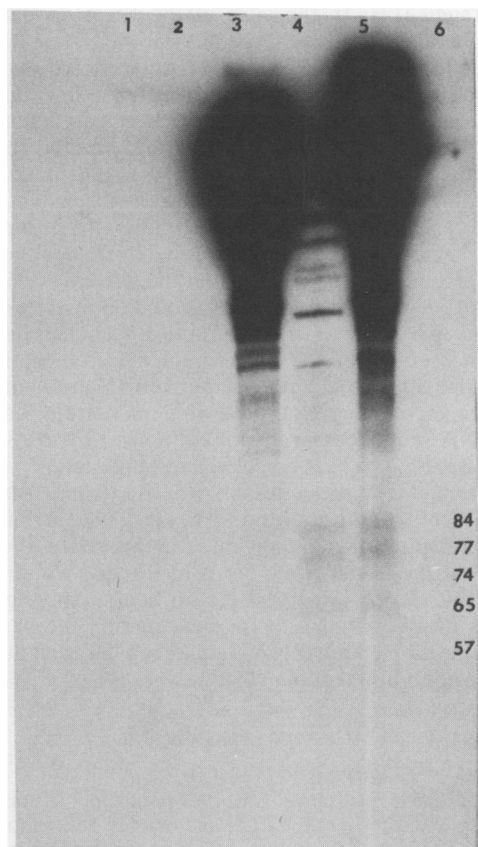


FIG. 6. Subcellular location of leader RNA-N protein RNPs. Cultures of infected cells were lysed and separated into S100 and P100 fractions (5). The fractions were immunoprecipitated with anti-N or anti-I-A<sup>p</sup> monoclonal antibodies and separated on 12% polyacrylamide gels containing urea. After electrotransfer, the filters were hybridized with a leader-specific cDNA probe (6), washed, and exposed to XAR-5 film at  $-70^{\circ}\text{C}$ . Lanes: 1, S100 anti-I-A; 2, P100 anti-I-A; 3, P100 anti-N; 4, S100 anti-N; 5, infected-cell control; 6, uninfected-cell control. Nucleotide numbers are shown on the right.

RNA was probed for the presence of negative-stranded RNA with in vitro-transcribed plus-sense viral RNAs. Negative-stranded RNA was immunoprecipitated with anti-N but not anti-E2 monoclonal antibody (Fig. 7), consistent with the suggestion that the N protein is associated with the transcription complex (12). These data cannot distinguish between N binding to negative-stranded RNA and the coimmunoprecipitation of the negative-stranded RNA as a component of the RI complex via N interactions with the leader-containing nascent plus-strands on the RI RNA. While it is possible that negative-stranded RNA associates with plus-strand RNA during extraction and immunoprecipitation, Sawicki and Sawicki (31) have shown that little, if any, free negative-stranded RNA is present in infected cells. This suggests that the negative-stranded RNA is coimmunoprecipitated as part of the RI complex.

The P100 and S100 subcellular fractions were also examined for the presence of N protein associated with negative-stranded RNA. Virtually all of the N-bound negative-stranded RNA (90%) was associated with membrane fractions by densitometric scanning of slot blots (Fig. 8). The small amount of negative-stranded RNA detected in the cytosol probably represents contaminating membrane

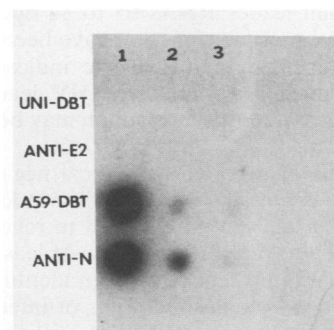


FIG. 7. Analysis of N-protein negative-stranded RNA interaction. Intracellular RNA was extracted from MHV-infected cells and coimmunoprecipitated with monoclonal antibodies directed against the MHV N or E2 structural proteins. The RNA was denatured, bound to nitrocellulose filters, and hybridized with radiolabeled plus-stranded RNA transcribed in vitro with the T7 RNA polymerase. Lanes 1 through 3, 10-fold dilutions of intracellular RNA. UNI-DBT, Uninfected DBT cells.

present during separation. These data support previous findings suggesting that free leader RNA-N complexes were present in the cytosol and were dissociated from the negative-stranded template. These data further support the contention that the N protein is associated with the membrane-bound transcription complex (9, 10).

## DISCUSSION

In this report, we have demonstrated a specific interaction between the MHV N protein and MHV viral mRNAs. Neither prelabeled host cell RNA, actin mRNA, nor VSV RNAs are associated with the MHV N protein. Previous studies in our laboratory have suggested that the leader RNA of MHV acts in *trans* as a primer for subgenomic mRNA synthesis, on the basis both of findings that leader RNA reassortment occurs at high frequency during MHV infection and of the presence of small leader RNAs in infected-cell extracts (4-6, 25, 32). Although the exact leader RNA which functions in virus transcription has not been identi-

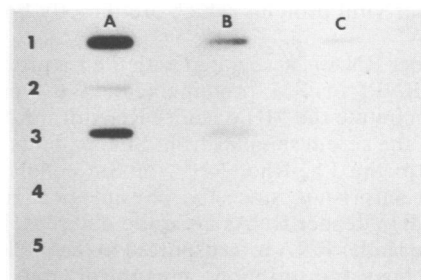


FIG. 8. Immunoprecipitation of MHV negative-stranded RNA from membrane and cytosolic fractions. Intracellular RNA was extracted from MHV-infected cells, separated into P100 and S100 fractions by ultracentrifugation, and precipitated with anti-N monoclonal antibody. The RNA was extracted, bound to nitrocellulose filters, and hybridized with plus-sense radiolabeled MHV RNAs transcribed in vitro with the T7 polymerase. Horizontal lanes: 1, anti-N-precipitated P100; 2, anti-N-precipitated S100; 3, intracellular RNA from MHV-infected cells; 4, purified MHV genomic RNA; 5, uninfected cells. Vertical lanes A through C, 10-fold dilutions of intracellular RNA.



fied, several small leader RNAs 65 to 84 nucleotides long which could serve a primer function have been identified (4, 5). The data presented in this article indicate that an N binding site is present within the MHV leader RNA sequence and suggest that this interaction may be important in viral transcription.

The N proteins of a variety of helical negative-stranded RNA viruses are essential for virus transcription. For example, the N protein of VSV is believed to regulate the shift from transcription to replication (2, 3). At least two distinct forms of the VSV N protein have been identified by monoclonal antibodies in cytoplasmic pools of infected cells (2). These studies imply that the availability of the unbound VSV N protein controls the rate of genomic RNA replication. The N proteins of VSV and La Crosse virus are also bound to the viral RNAs in an RNA complex (17, 28, 30); however, the function of these complexes in virus replication is unclear. Coronaviruses represent the only plus-polarity RNA animal viruses with helical nucleocapsids (38). Compton et al. (12) have clearly shown that the N protein of MHV functions in viral RNA synthesis, since anti-N monoclonal antibody inhibits *in vitro* transcription. Similar results have been demonstrated in our laboratory (P. R. Brayton et al., unpublished observation). However, the nature of the interaction between N and the transcriptional complex is unclear. Our findings suggest that N is bound to the cytosol- and membrane-bound small leader RNAs 65 to 84 nucleotides long as well as to the transcription complex. Although the exact leader RNA which functions in viral transcription has not been identified, it is clear that *trans*-acting leader RNAs function during MHV subgenomic RNA synthesis and that the minimum size of this RNA is approximately 65 nucleotides (25, 32). The data presented in this paper suggest that such an RNA will contain a functional N binding site, suggesting that a *trans*-acting leader RNA-N complex may function during subgenomic mRNA synthesis. Immunoprecipitation of the negative-stranded RNA, as well as the small leader RNAs originating during mRNA 6 transcription, also suggests that N is associated with the transcription complex. Since the nascent plus-strands on the RI RNA contain leader RNA (4, 5), it seems likely that N protein binding is mediated at least in part via these sequences. These data suggest that N may play an important structural role in the transcription complex; however, our data cannot exclude the possibility that the N protein is complexed with negative-stranded RNA or with other viral proteins which are directly bound to the leader RNA.

VSV leader RNA is associated with the La protein but not the Rho, RNP, or SM proteins (20). We were able to immunoprecipitate the MHV leader RNA or mRNA species with any of the serum samples from SLE patients containing antibodies to the La, Rho, RNP, or Sm cellular proteins. This is not surprising, since the physiological roles of the VSV and MHV leader RNAs are quite different. In the case of VSV, the leader RNA is transported to the nucleus, where it inhibits host transcription, presumably by binding to cellular La protein (3, 20). Conversely, MHV probably inhibits the host cell macromolecular synthesis at the level of translation (19), while leader RNA probably functions as a primer for transcription of the subgenomic mRNAs (4-6, 24).

The VSV leader RNA is also bound to the nucleocapsid protein in an RNP complex (3, 7). Since the leader RNA sequences of VSV are also present in genomic RNA, but not mRNA, it is thought that these sequences regulate encapsidation of genome into RNP structures. Interestingly, the VSV mRNAs are also detected in RNP complexes but are

not packaged (17). In the case of the MHV leader RNA, these sequences are present on all viral mRNAs, suggesting that other factors are involved in regulating the encapsidation of the genomic RNA, but not mRNA, into infectious virions. Encapsidation of viral genomic RNAs is a complex process frequently requiring one or more binding sites (26). The exact role of the leader sequence in encapsidation of the MHV genome is currently under study.

Presently, the exact nucleotides within the leader RNA sequence which bind N have not been determined; however, the data presented here suggest that the site may reside 3' of nucleotide 57, since we were unable to demonstrate that the small leader RNAs of 47, 50, and 57 nucleotides were present in infected cells as RNP complexes. Alternatively, N may require a minimum RNA length for high-affinity binding. In the accompanying article we demonstrate that MHV leader RNA sequences, transcribed *in vitro* by the T7 RNA polymerase, bind to N in a specific and high-affinity fashion, while internal MHV sequences in gene A (genome RNA) and gene F (mRNA 6) do not bind to N (35). Together with the immunoprecipitation data presented in this study, these data suggest that the leader RNA contains a sequence-specific N binding site. More importantly, the accompanying article describes a novel approach for determining the N binding site within the leader RNA sequences as well as other regions in the viral genome (35).

#### ACKNOWLEDGMENTS

We thank J. Goodnight, K. Trass, P. Driscoll, and L. Pen for excellent technical assistance, Carol Flores for preparation of the manuscript, and Michael M. C. Lai and Stanley Tahara for helpful discussions.

This work was supported by National Science Foundation grant DMB-87-17148, American Heart Association grant 40073, and Public Health Service grants AI 31386, AI 19244, NS 18146, NS 00796, and AI 23946 from the National Institutes of Health.

#### LITERATURE CITED

1. Armstrong, J., S. Smeekens, and P. Rottier. 1983. Sequence of the nucleocapsid gene from murine coronavirus MHV-A59. *Nucleic Acids Res.* 11:883-891.
2. Arnheiter, H., N. L. Davis, G. Wertz, M. Schubert, and R. A. Lazzarini. 1985. Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. *Cell* 41:259-267.
3. Banerjee, A. 1987. Transcription and replication of rhabdoviruses. *Microbiol. Rev.* 51:66-87.
4. Baric, R. S., C.-K. Shieh, S. A. Stohlman, and M. M. C. Lai. 1987. Analysis of intracellular small RNAs of mouse hepatitis virus: evidence for discontinuous transcription. *Virology* 156:342-354.
5. Baric, R. S., S. A. Stohlman, and M. M. C. Lai. 1983. Characterization of replicative intermediate RNAs of mouse hepatitis virus: presence of leader RNA sequences on nascent chains. *J. Virol.* 48:633-640.
6. Baric, R. S., S. A. Stohlman, M. K. Razavi, and M. M. C. Lai. 1985. Characterization of the leader-related small RNAs in coronavirus-infected cells: further evidence for leader-primed mechanism of transcription. *Virus Res.* 3:19-33.
7. Blumberg, B. M., C. Giorgi, and D. Kolakofsky. 1983. N protein of vesicular stomatitis virus selectively encapsidates leader RNA *in vitro*. *Cell* 32:559-567.
8. Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub. 1980. The detection of DNA-binding proteins by protein blotting. *Nucleic Acids Res.* 8:1-20.
9. Brayton, P. R., M. M. C. Lai, C. D. Patton, and S. A. Stohlman. 1982. Characterization of two RNA polymerase activities induced by mouse hepatitis virus. *J. Virol.* 42:847-853.
10. Brayton, P. R., S. A. Stohlman, and M. M. C. Lai. 1984. Further characterization of mouse hepatitis virus RNA-dependent RNA

- polymerases. *Virology* 133:197-201.
11. Chou, C. C., R. C. Davis, M. L. Fuller, J. P. Slovin, A. Wong, J. Wright, S. Kania, R. Shaked, R. A. Gatti, and W. A. Salser. 1987. Gamma-actin: unusual mRNA 3'-untranslated sequence conservation and amino acid substitutions that may be cancer related. *Proc. Natl. Acad. Sci. USA* 84:2575-2579.
  12. Compton, J. R., D. B. Rogers, K. V. Holmes, D. Fertsch, J. Remenick, and J. J. McGowan. 1987. In vitro replication of mouse hepatitis virus strain A59. *J. Virol.* 61:1814-1820.
  13. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
  14. Fleming, J. O., S. A. Stohman, R. C. Harmon, M. M. C. Lai, and J. A. Frelinger. 1983. Antigenic relationship of murine coronavirus: analysis using monoclonal antibodies to JHM (MHV-4) virus. *Virology* 131:296-307.
  15. Fried, M. G., and D. M. Crothers. 1984. Equilibrium studies of the cyclic AMP receptor protein-DNA interaction. *J. Mol. Biol.* 172:241-262.
  16. Gilmore, W., J. O. Fleming, S. A. Stohman, and L. P. Weiner. 1987. Characterization of the structural proteins of the murine coronavirus strain A59 using monoclonal antibodies. *Proc. Soc. Exp. Biol. Med.* 185:177-186.
  17. Grubman, M. J., and D. A. Shafritz. 1977. Identification and characterization of messenger ribonucleoprotein complexes from vesicular stomatitis virus-infected HeLa cells. *Virology* 8: 1-16.
  18. Harmon, R. C., N. Stein, and J. A. Frelinger. 1983. Monoclonal antibodies reactive with H-2 determinants. *Immunogenetics* 18: 541-545.
  19. Hilton, A., L. Mizzen, G. MacIntyre, S. Cheley, and R. Anderson. 1986. Translational control in murine hepatitis virus infection. *J. Gen. Virol.* 67:923-932.
  20. Kurilla, M. G., and J. D. Keene. 1983. The leader RNA of vesicular stomatitis virus is bound by a cellular protein reactive with anti-La lupus antibodies. *Cell* 34:837-845.
  21. Lai, M. M. C., R. S. Baric, P. R. Brayton, and S. A. Stohman. 1984. Characterization of leader RNA sequences on the virion and mRNAs of mouse hepatitis virus, a cytoplasmic RNA virus. *Proc. Natl. Acad. Sci. USA* 81:3626-3630.
  22. Lai, M. M. C., C. D. Patton, and S. A. Stohman. 1982. Further characterization of mRNAs of mouse hepatitis virus: presence of common 5'-end nucleotides. *J. Virol.* 41:557-565.
  23. Lai, M. M. C., and S. A. Stohman. 1978. RNA of mouse hepatitis virus. *J. Virol.* 26:236-242.
  24. Makino, S., N. Fujioka, and K. Fujiwara. 1985. Structure of the intracellular defective viral RNAs of defective interfering particles of mouse hepatitis virus. *J. Virol.* 54:329-339.
  25. Makino, S., S. A. Stohman, and M. M. C. Lai. 1986. Leader sequences of murine coronavirus mRNAs can be freely reassorted: evidence for the role of free leader in RNA transcription. *Proc. Natl. Acad. Sci. USA* 83:4204-4208.
  26. Mann, R., and D. Baltimore. 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. *J. Virol.* 54:401-407.
  27. Nakanaga, K., K. Yamanouchi, and K. Fujiwara. 1986. Protective effect of monoclonal antibodies on lethal mouse hepatitis virus infection in mice. *J. Virol.* 59:168-171.
  28. Raju, R., and D. Kolakofsky. 1987. Unusual transcripts in La Crosse virus-infected cells and the site of nucleocapsid assembly. *J. Virol.* 61:667-672.
  29. Robbins, S. G., M. F. Frana, J. J. McGowan, J. F. Boyle, and K. V. Holmes. 1986. RNA-binding proteins of MHV: detection of monomeric and multimeric N protein with an RNA overlay-protein blot assay. *Virology* 150:402-410.
  30. Rosen, C. A., H. L. Ennis, and P. S. Cohen. 1982. Translational control of vesicular stomatitis virus protein synthesis: isolation of an mRNA-sequestering particle. *J. Virol.* 44:932-938.
  31. Sawicki, S. G., and D. L. Sawicki. 1986. Coronavirus minus-strand RNA synthesis and effect of cycloheximide on coronavirus RNA synthesis. *J. Virol.* 57:328-334.
  32. Shieh, C.-K., L. H. Soe, S. Makino, M.-F. Chang, S. A. Stohman, and M. M. C. Lai. 1987. The 5'-end sequences of murine coronavirus genome: implications for multiple fusion sites in leader-primed transcription. *Virology* 156:321-330.
  33. Skinner, M. A., and S. G. Siddell. 1983. Coronavirus JHM: nucleotide sequence of the mRNA that encodes the nucleocapsid protein. *Nucleic Acids Res.* 11:5045-5054.
  34. Spaan, W. J. M., P. J. M. Rottier, M. C. Horzinek, and B. A. M. van der Zeijst. 1981. Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). *Virology* 108:424-434.
  35. Stohman, S. A., R. S. Baric, G. N. Nelson, L. H. Soe, L. M. Welter, and R. J. Deans. 1988. Specific interaction between coronavirus leader RNA and nucleocapsid protein. *J. Virol.* 62: 4288-4295.
  36. Stohman, S. A., J. O. Fleming, C. D. Patton, and M. M. C. Lai. 1983. Synthesis and subcellular localization of the murine coronavirus nucleocapsid protein. *Virology* 130:527-532.
  37. Stohman, S. A., and M. M. C. Lai. 1979. Phosphoproteins of murine hepatitis viruses. *J. Virol.* 32:672-675.
  38. Sturman, L. S., and K. V. Holmes. 1983. The molecular biology of coronaviruses. *Adv. Virus. Res.* 28:35-112.
  39. Sturman, L. S., K. V. Holmes, and J. Behnke. 1980. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J. Virol.* 33:449-462.
  40. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments to nitrocellulose. *Proc. Natl. Acad. Sci. USA* 77:5201-5205.
  41. Xi, J., M. K. Estes, and T. G. Metcalf. 1987. Detection of hepatitis A virus by hybridization with single-stranded RNA probes. *Appl. Environ. Microbiol.* 53:2487-2495.